PUBLIC TRANSLATION INTO ENGLISH.----------

Descriptive Memory.----------

Of the Invention Patent----------

On----------

Genetic constructions, cloning methods of the gene encoding for Recombinant Human Erythropoietin, selection of producing cell lines and cell cultures for the mass production of Recumbent Human Erythropoietin.----------

Aplied by:----------

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For the term of 20 years.----------

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I. Technical description of the invention----------

The present invention relates to gene constructions, cloning methods of the gene encoding for Recombinant Human Erythropoietin, selection of producing cell lines and cell cultures for the mass production of Recombinant Human Erythropoietin.----------

II. Technical field of the invention----------

Genomic DNA that leads to EPO production has been reduced in the present invention to its minimum expression, including only encoding sequences and the intros comprised by said sequences plus a few bases at 3' from the stop codon, and excluding any homologous sequence, either regulatory or not, at 5' from the initial ATG. The prior art states that the sequences hereby excluded are very important in order to obtain a better protein productivity.----------

The vectors used in this invention, in contrast to those described to date for EPO mass production, have extremely simple expression

control genetic elements, and allow a double selection of the recombinant cells with antibiotics and methotrexate.----------

These elementary genetic constructions unexpectedly make the cells transfected with them, produce surprisingly high quantities of EPO per ml of culture medium per day, several times superior to those described in former patents and publications. ----------

Novelty of the invention – Purpose of the patent----------

The genetic constructions described to present for EPO production invariably use homologous elements located at 5' from the first translated ATG codon, explaining in many cases that this presence of multiple control elements markedly facilitates the protein expression.

The genomic DNA used in the present invention patent to produce EPO was isolated so as not to use any homologous element located at 5' from the firs translated ATG codon, so as to allow the expression control genetic elements located in the plasmids used, operate in a similar way as they naturally do, that is to say, operating directly on a codifying gene, without foreign sequences acting between this gene and the very control sequences.----------

In this way, the combination of control elements and the gene encoding for EPO, purpose of this patent, operate with high efficiency, thus achieving high EPO expressions, comparable to and even higher than those reported by using much more complex and difficult to manipulate genetic constructions.----------

Additionally, cotransfection with the two vectors described herein, which confer different resistances, facilitates the selection, genetic amplification and maintenance of the cotransfected producing cells.----

Example 1 ----------

To obtain genetic constructions the following procedure was

performed:----------

Preparation of Human Genomic DNA.----------

10 ml of blood in 10 mM EDTA (pH 8) were extracted from a clinically healthy adult male subject. The blood was transferred in 5 ml aliquots to two 50 ml tubes, to which 45 ml of a solution containing 0.3 M of saccharose, 10 mM Tris-HCL (pH 7.5), 5 mM Mg Cl_2 and 1% of Triton X 100 was added. The resulting solution was stored at 4° C.----------

The solution was then placed on ice for 10 minutes and centrifuged for 10 minutes at 1000 g and at 4°C. The supernatant was discarded and the pellet was washed up several times with a 0.075 M NaCl solution containing 0.025 M EDTA (pH 8), followed by centrifugation for 10 minutes at 1000 g and at 4°C.----------

The resulting pellet thus obtained was resuspended in 3 ml of a 10mM Tris-HCl (pH 8), 400 mM NaCl, 2 mM EDTA (pH 8) solution. 200 µl of 10 % SDS (sodium dodecyl sulphate) and 500 µl K proteinase (1 mg/ml in 1 % SDS and 2 mM EDTA pH 8) were then added, and the solution was incubated overnight at 37°C. After this incubation, 1 ml of NaCl saturated solution was added; the solution was shaken and then centrifuged at 2500 g for 15 minutes.----------

The supernatant was transferred to a 15 ml tube where the volume was duplicated by the addition of isopropanol. The supernatant and isopropanol were gently mixed by inversion of the tube and stored at room temperature until a DNA precipitate was formed, which was "fished" with a Pasteur blent glass pipette.----------

The DNA was placed in a 2 mL tube, and 1 mL of 70 % ethanol was added. After the solution was left stand for one minute, the supernatant was discarded and the precipitate was left to dry. After drying, the precipitate was suspended in 500 µl of TE (10 mM Tris-

HCl pH 8 - 1 mM EDTA).----------

Concentration of DNA solution was calculated by measuring the absorbance at 260 nm of a 1:1000 dilution of this solution. Each unit of optical density was considered to have 50 µg of genomic DNA. Once the concentration was known, a solution was prepared with 500 ng of genomic DNA per µl in TE.----------

B. Preparation of the EPO encoding gene, proper for its expression.----------

Gene encoding for EPO was prepared from 500 ng of human genomic DNA obtained in Example 1, adding 400 ng of each of the EPO 1 and EPO 2 primers, in an aqueous 2.5 mM solution containing each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 2.5 units of Taq DNA polymerase (Perkin Elmer) in a final volume of 100 µl using the buffer recommended by the manufacturer. A Perkin Elmer-Cetus Thermal Cycler 480 was used and was programmed for 30 cycles of: 1 minute at 93□C, 1 minute at 55□C and 3 minutes at 72□C. From this reaction, a fragment of approximately 2170 bases containing the EPO gene was obtained.----------

The sequence of olygonucleotides employed was:----------

EPO 1: 5' GAATTCTCGAGATGGGGGTGCACGGTGAG 3' (SEQ No:2), corresponding to the first bases translated from the EPO gene with the addition of a recognition site for the Xho I enzyme and one for the recognition of Eco RI enzyme to the 5' end. These sites were used in the subsequent cloning steps.----------

EPO 2: 5' AAGCTTGGACACACCTGGTCATCTG 3' (SEQ No.:3), complementary to the last translated bases and to some of the non-encoding 3' of the EPO gene. A site for the recognition of the Hind III enzyme was added to the 3' end. This site was used in subsequent

cloning steps.----------

The sequence obtained is as follows (SEQ No:4):----------

gaattctcgagatgggggtgcacggtgagtactcgcgggctgggcgctcccgccgcccgggtc
cctgtttgagcggggatttagcgccccggctattggccaggaggtggctgggttcaaggaccggc
gacttgtcaaggaccccggaaggggggaggggggtggggcagcctccacgtgccagcgggga
cttgggggagtccttggggatggcaaaaacctgacctgtgaaggggacacagtttgggggttga
ggggaagaaggtttgggggttctgctgtgccagtggagaggaagctgataagctgataacctgg
gcgctggagccaccacttatctgccagaggggaagcctctgtcacaccaggattgaagtttggc
cggagaagtggatgctggtagctgggggtggggtgtgcacacggcagcaggattgaatgaag
gccagggaggcagcacctgagtgcttgcatggttggggacaggaaggacgagctggggcag
agacgtggggatgaaggaagctgtccttccacagccacccttctccctccccgcctgactctcag
cctggctatctgttctagaatgtcctgcctggctgtggcttctcctgtccctgctgtcgctccctctgggc
ctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctggagaggtacctctt
ggaggccaaggaggccgagaatatcacggtgagacccccttccccagcacattccacagaact
cacgctcagggcttcagggaactcctcccagatccaggaacctggcacttggtttggggtggagt
tgggaagctagacactgccccccctacataagaataagtctggtggccccaaaccatacctgga
aactaggcaaggagcaaagccagcagatcctacggcctgtgggccagggccagagccttca
gggacccttgactccccgggctgtgtgcatttcagacgggctgtgctgaacactgcagcttgaatg
agaatatcactgtcccagacaccaaagttaatttctatgcctggaagaggatggaggtgagttcct
ttttttttttttttccttttcttttggagaatctcatttgcgagcctgattttggatgaaagggagaatgatcgg
gggaaaggtaaaatggagcagcagagatgaggctgcctgggcgcagaggctcacgtctataa
tcccaggctgagatggccgagatgggagaattgcttgagccctggagtttcagaccaacctagg
cagcatagtgagatcccccatctctacaaacatttaaaaaaattagtcaggtgaagtggtgcatg
gtggtagtcccagatatttggaaggctgaggcgggaggatcgcttgagcccaggaatttgaggct
gcagtgagctgtgatcacaccactgcactccagcctcagtgacagagtgaggccctgtctcaaa
aaagaaaagaaaaaagaaaaataatgagggctgtatggaatacattcattattcattcactcact
cactcactcattcattcattcattcaacaagtcttattgcataccttctgtttgctcagcttggtgctt
ggggctgctgaggggcaggagggagagggtgacatgggtcagctgactcccagagtccactc

cctgtaggtcgggcagcaggccgtagaagtctggcagggcctggccctgctgtcggaagctgtc
ctgcggggccaggccctgttggtcaactcttcccagccgtgggagcccctgcagctgcatgtgga
taaagccgtcagtggccttcgcagcctcaccactctgcttcgggctctgggagcccaggtgagta
ggagcggacacttctgcttgccctttctgtaagaaggggagaagggtcttgctaaggagtacagg
aactgtccgtattccttccctttctgtggcactgcagcgacctcctgttttctccttggcagaaggaag
ccatctcccctccagatgcggcctcagctgctccactccgaacaatcactgctgacactttccgca
aactcttccgagtctactccaatttcctccggggaaagctgaagctgtacacaggggaggcctgc
aggacaggggacagatgaccaggtgtgtcc***aagctt*** ----------

The first translated atg codon, as well as the tga "stop" codon are underlined. ----------

The sequences of restriction sites.----------

C. Cloning and sequencing.----------

A fragment of approximately 2170 base pairs corresponding to the EPO gene was purified and the ends were blunted by treatment with the RNA polymerase Klenow fragment and cloned in the Sma I site of a M13mp18 vector, following standard techniques applied in molecular biology. The recombinant plasmids obtained were cut with the Xho I and Hind III enzymes; the presence of the insert was verified by electrophoresis of the product resulting from the restriction fragments in a 0.8 % agarose gel developed with ethydium bromide stain. A positive clone (two bands, one having approximately 2200 base pairs and the other one corresponding to the linear vector) was chosen and manually sequenced according to the Sanger's technique using a "T7 sequencing kit" (Pharmacia) and with the aid of an automatic sequences Model 370 A Applied Biosystems International. For each sequencing system the protocols recommended by the manufacturers were followed.----------

D. pVex 1 and pDHFR Vectors.----------

1. Construction of pVex 1 Vector.----------

The pVex1 vector was built following the conventional techniques used in molecular biology. It consisted of:----------

a. Fragments of the bacteria1 pBR322 vector, which conferred a bacterial replication origin and resistance to ampicillin, for amplification and selection of the vector in *E. coli*.----------

b. Immediately close to 3' of a) an early promoter of the SV40 virus is located, which allows the expression of the genes cloned at 5' from this element.----------

c. Immediately close to 3' of b) the Xho I and Hind III cloning sites are located, which allow insertion of the genes to be expressed.----------

d. Immediately close to 3' of c) the polyadenylation signal of the SV40 virus is located, which allows the proper polyadenylation of the specific transcripts of the gene cloned in c).----------

e. Immediately close to 3' of d) the TK promoter and the gene coding for neomycin phosphotransferase plus the polyadenylation signal are located to allow the selection of stably transfected cells through selection by resistance to neomycin and neomycin-derived antibiotics such as genetycin. The 3' end of e) is linked to the 5' end of a).----------

2. pDHFR Vector----------

The pDHFR vector confers resistance to ampicillin to aid in selection in bacteria. and includes the DNA copy encoding for mice dehydrofolate reductase (DHFR), whose expression is controlled by the SV40 virus early promoter and its polyadenylation signal.----------

The coexpression of DHFR and the EPO-encoding gene allows, through selection by adding methotrexate (MTX) to the culture medium, several times-amplification of EPO expression achieved with

the pVex 1-EPO vector.----------

E. Cloning of the EPO-Encoding gene into an Expression Vector-

The sequenced gene was removed by cleavage with the Xho I-Hind III enzymes of the vector where it was cloned in Example 3. It was then isolated and cloned in the same restriction sites of the pVex I vector. - A positive pVex-EPO clone was isolated. All these operations were performed according to the conventional genetic engineering techniques.----------

F. Co-transfection and amplification with MTX.----------

A CHO (Chinese Hamster Ovary) cell line, mutated to be deficient in the DHFR-enzyme gene (CHO-DHFR), was used to facilitate the genetic amplification with MTX. ----------

During this whole process, cells were grown at 37□ C in a 5% of $C0_2$ atmosphere.----------

CHO cells were cotransfected following the calcium phosphate technique which, for a 90 mm-diameter Petri dish, consists in:----------

(a) Replacing the culture medium (alpha-MEM, with10 % of bovine fetal serum) with fresh medium 4-8 hours before transfection.----------

(b) Adding to a 5 mL tube a 10 g/l HEPES solution (pH 7.1), 16 g/l NaCl and 10 µl of a 35 mM Na_2HPO_4 and 35 mM of NaH_2PO_4 solution.----------

(c) Preparing in a separate 1.5 ml tube a solution with 60 µl of 2 M $CaCl_2$ and 10 µg of each DNA to be transfected (pVex-EPO and pDHFR). Water was added until the volume reached 500 µl. The pDHFR plasmid described in Example b is based on the pBR 322 plasmid, which confers resistance to ampicillin, can replicate in *E.coli*, has the DHFR gene cloned between the early promoter and the terminator of the SV40, and allows the expression of the DHFR protein

in CHO cells. This protein confers resistance to methotrexate, which can be then used to select cells having a high erythropoietin productivity.----------

(d) Adding drop by drop, the solution containing DNA and $CaCl_2$ to the tube containing Hepes, while air is bubbled to obtain a rapid mixing and to make the local concentrations as small as possible. This method facilitates the formation of a very thin precipitate which is more effectively incorporated by the cells. ----------

(e) The solution is allowed to settle for 30 minutes and then is poured on the cells.----------

(f) The solution is distributed among the cells through gentle shaking, and left overnight in an incubator at 37° C under a 5% CO_2 atmosphere.----------

(g) The cells are washed twice with PBS (8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4, 0.24 g NaH_2PO_4, water is added until the volume reached 1 liter and pH is adjusted to 7.4 with HCl. Fresh culture medium is then added.----------

Twenty four hours after transfection, the selection with genetycin (G 418) at a final concentration of 600 µg/ml was begun. The cells that stably incorporated the pVex-EPO plasmid were able to resist the antibiotic while all the others died after 25 days. Resistant colonies were selected and their productivity was assayed. Once the clones were isolated, the three most productive ones were selected.----------

Taking advantage of the genetic constructions used in the invention, a selection was performed with each of the three clones using a second selective agent: MTX at different concentrations: 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M. For that purpose, the culture medium was changed to alpha-MEM without nucleosides, supplemented with 10% dialyzed bovine

fetal serum. It was essential to perform the dialysis process according to the following schedule: for 100 ml of serum, the serum is placed in a dialysis bag with a porosity under 3000 Da (with a higher porosity, growth factors would be lost, and the cells would not be able to grow and reproduce), the bag is hermetically closed, and completely immersed in a recipient with 5 liters of bidistilled water; where it is left at 4°C for 12 hours. After this, the water is discarded and again 5 liters are added and the bag is then left stand at 4°C for an additional 12 hour period. Then the dialysis bag is removed and the serum is recovered. Dialysis during shorter periods or with smaller volumes, or without replacing the water, would be worthless since a small amount of nucleotides could be left in serum, and therefore the selection with MTX would not work. Dialysis during longer periods would also be worthless since some proteins, necessary for cell growth, could precipitate and be lost.----------

G. Isolation of mass production cell lines----------

Clones that grew in 10^{-7} and 10^{-6} M of MTX were isolated, amplified in fresh alpha-MEM without nucleosides supplemented with 10% of dialyzed bovine fetal serum. Once grown, the culture supernatant was assayed to measure EPO production and secretion. For that purpose, a specific immunoassay was used.----------

The process described above concluded with the selection of a clone of recombinant cells producing 50,000 µg of erythropoietin/liter of culture medium per day.----------

Cell transcripts specific for EPO were controlled as described in H, to verify that there were no mistakes in the DNA sequence used or in its transcription. ----------

In order to identify the protein obtained, it was proceeded as described

in I.----------

H. Verification of the Specific messenger RNA Sequence Produced by the Recombinant Cells.----------

1.. Preparation of RNA from cells.----------

Total RNA was prepared from producing cell lines, according to the following protocol:----------

- 90-mm diameter Petri dish with confluent cells was washed twice with 10 ml of PBS.----------

- Two ml of GTC buffer were then added and spread all over the dish. The GTC buffer was composed of: (1) 50 g guanidinum thiocyanate; (2) 0.5 g N-Lauroilsarcosin; (3) 2.5 ml 1 M sodium citrate, pH 7; (4) 0.7 ml β-mercapthoethanol; (5) 0.33 ml 30% antifoam agent (SIGMA); (6) H_2O q.s.. 100 ml, pH 7.0.----------

Cells were lysed resulting in a highly viscous solution. The solution was transferred to a 15 ml tube, and the process above described was repeated once more using 2 ml of GTC buffer.----------

- The 15 ml tube was vigorously shaken for 1 minute to break the DNA. Fractioning in a cesium chloride gradient was then performed-.- For that purpose, 4 ml of a solution containing CsCl (95.97 g CsCl and 2.5 ml of 1 M Sodium Acetate, pH 5.4, water was added to reach a volume of 100 ml) were poured in an ultracentrifuge tube. Over this solution and without mixing, the suspension of the cells in GTC was added. The tube was then filled with GTC buffer and ultracentrifuged at 20°C, for 20 hours at 31000 rpm.----------

- The RNA remained at the bottom of the tube (pellet) and the DNA formed a band in the middle of the cesium chloride gradient. ----------

- The supernatant was discarded, taking special care to eliminate all of the DNA. The RNA-containing pellet was left to dry for 5 minutes.------

- The pellet was dissolved in 200 ml of water and transferred to a 1.5 ml tube.----------

- 200 ml of 0.4 M Sodium Acetate, pH 4.8, and 2 volumes of ethanol were then added, the resulting solution was thoroughly mixed and left to stand for 30 minutes at –80°C. ----------

- The solution was then centrifuged in a microcentrifuge at 14000 rpm for 15 minutes, the supernatant was discarded and the precipitate was rinsed with 1 ml of 80 % ethanol. ----------

- The pellet was dried and redissolved in 100 ml of water. ----------

- The concentration of a 1:100 dilution of the RNA solution was measured at 260 nm (one optical density unit is equivalent to 40 mg of RNA). ----------

NOTE: All the solutions and elements used were RNAase-free.----------

2. Preparation of specific cDNA.----------

Specific cDNA was prepared following the directions of a kit intended for that purpose (cDNA Synthesis System Plus, Amersham - cat. RPN 1256). EPO2 oligonucleotide was used as specific the primer.----------

3. Cloning of cDNA Encoding for EPO.----------

1/20th of the obtained cDNA was amplified using 400 ng of each the EPO2 and EPO3 oligonucleotides, and 2.5 mM of each deoxynucleotide in the proper buffer, and 2.5 units of Taq DNA polymerase, in a total volume of 100 ml.----------

Thirty five amplification cycles were performed as follows: 1 minute at 93°C, 1 minute at 55°C and 1 minute at 72°C.----------

EPO 3 was synthesized as described for **EPO 1** and **EPO-2**, and its sequence (5' GAATTCCATGGGGGTGCACGAATGTCC 3') (SEQ ID NO:5) corresponded to the first 20 bases encoding for the EPO cDNA, adding one site for the recognition of the Eco RI enzyme, to facilitate

the subsequent genetic manipulations.----------

A fragment of approximately 600 base pairs was obtained, which was cloned in M13mp18 and M13mp19 vectors. ----------

The presence of the insert in the clones with restriction fragments was assayed and sequenced in both directions to obtain the complete sequence, using the Sanger's method.----------

Due to the very high autocomplementarity of some regions of the gene, which gives rise to many and very ambiguous compressions in the radioautography, a sequencing kit using Taq DNA polymerase and modified bases was used. Lower quality results were obtained, but the compressions were eliminated. The kit used was the Pharmacia-LKB Biotechnology *Gene aTaq*----------.

The complete sequence of the human erythropoietin DNA copy, isolated and cloned, showed to encode for EPO. Therefore, no mistakes in the gene or in its transcription were possible.----------

I. Study of the EPO Produced.----------

The EPO obtained by culturing the cells of this example underwent different quality and identification assays.----------

1. In a denaturing SDS-PAGE gel the EPO was identified as a wide band of molecular weight superior to 30 kDa. See Figure 1----------

2. The band was recognized by a monoclonal antibody and by a polyclonal antibody to human EPO in a "Western blot" assay. See Figure 2.----------

3.Treatment with glycanases proved the existence of the glycosidic chains whose quantity and molecular weight were as expected. See Figure 3.----------

4.The EPO produced proved to be composed of a series of species with isoelectric points ranging from 3.0 to 4.5. ----------

5.The complete amino acid sequence of the isolated protein, purified from the culture supernatant of transfected cell lines showed total homology with natural human erythropoietin whose 165 aminoacid sequence is as follows (SEQ No:1):----------

NH_2--- Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg

Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys

Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala

Glu Hys Cys Ser Leu Asn Glu Asn Ile Thr

Val Pro Asp Thr Lys Val Asn Phe Tyr Ala

Trp Lys Arg Met Glu Val Gly Gln Gln Ala

Val Glu Val Trp Gln Gly Leu Ala Leu Leu

Ser Glu Ala Val Leu Arg Gly Gln Ala Leu

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro

Leu Gln Leu Hys Val Asp Lys Ala Val Ser

Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg

Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser

Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu

Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys

Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala

Sys Arg Thr Gly Asp Arg---- COOH .----------

X Glycosilation sites.----------

6.The presence of the four glycosilation sites on the 165 amino acid chain, as well as the complex carbohydrate structure, specifically, the sialic acid terminal residues, were demonstrated, as well as its correct *in vivo* biological activity, when assayed by the model of the ex-hypoxic polycythemic mouse assay, which showed a total parallelism versus the corresponding international standard.----------

Productivity achieved, measured by a specific immunoassay, was 50 mg per liter of culture per day.----------

What Is Claimed Is:----------

Having described and exemplified the nature and main subject of this invention, as well as the manner in which it can be operated, it is hereby stated to claim as of exclusive property and rights:----------

1) GENETIC CONSTRUCTIONS, CLONING METHODS OF THE GENE ENCODING FOR RECOMBINANT HUMAN ERYTHROPOIETIN, SELECTION OF PRODUCING CELL LINES AND CELL CULTURES FOR THE MASS PRODUCTION OF RECOMBINANT HUMAN ERYTHROPOEITIN. characterized by the following: a) the genetic constructions use only the EPO human gene encoding region, without including homologous genenetic elements located at 5' from the first translated ATG codon; b) genetic constructions have viral promoters and terminators, as expression control systems; c) the cloning method for the codifing gene uses directly genomic DNA; d) the cell lines are selected using a double system, I) resistance to genetycin and

II) resistance to increasing quantities of Methotrexate and e) selected cells EPO productivity is larger than 50 mg/liter of culture medium/day.----------

The sequence used is as follows:----------

gaattctcgagatgggggtgcacggtgagtactcgcgggctgggcgctcccgccgc
ccgggtccctgtttgagcggggatttagcgccccggctattggccaggaggtggctgg
gttcaaggaccggcgacttgtcaaggaccccggaaggggggagggggtggggca
gcctccacgtgccagcggggacttgggggagtccttggggatggcaaaaacctgac
ctgtgaaggggacacagtttgggggttgaggggaagaaggtttgggggttctgctgtg
ccagtggagaggaagctgataagctgataacctgggcgctggagccaccacttatct
gccagaggggaagcctctgtcacaccaggattgaagtttggccggagaagtggatg
ctggtagctgggggtggggtgtgcacacggcagcaggattgaatgaaggccaggg
aggcagcacctgagtgcttgcatggttggggacaggaaggacgagctggggcaga
gacgtggggatgaaggaagctgtccttccacagccacccttctccctccccgcctga
ctctcagcctggctatctgttctagaatgtcctgcctggctgtggcttctcctgtccctgctg
tcgctccctctgggcctcccagtcctgggcgccccaccacgcctcatctgtgacagcc
gagtcctggagaggtacctcttggaggccaaggaggccgagaatatcacggtgag
acccccttccccagcacattccacagaactcacgctcagggcttcagggaactcctcc
cagatccaggaacctggcacttggtttggggtggagttgggaagctagacactgccc
ccctacataagaataagtctggtggccccaaaccatacctggaaactaggcaagga
gcaaagccagcagatcctacggcctgtgggccagggccagagccttcagggaccc
ttgactccccgggctgtgtgcatttcagacgggctgtgctgaacactgcagcttgaatg
agaatatcactgtcccagacaccaaagttaatttctatgcctggaagaggatggaggt
gagttccttttttttttttttccttttctttttggagaatctcatttgcgagcctgattttggatgaaa
gggagaatgatcgggggaaaggtaaaatggagcagcagagatgaggctgcctgg
gcgcagaggctcacgtctataatcccaggctgagatggccgagatgggagaattgc
ttgagccctggagtttcagaccaacctaggcagcatagtgagatcccccatctctaca
aacatttaaaaaaattagtcaggtgaagtggtgcatggtggtagtcccagatatttgga

aggctgaggcgggaggatcgcttgagcccaggaatttgaggctgcagtgagctgtg
atcacaccactgcactccagcctcagtgacagagtgaggccctgtctcaaaaaaga
aaagaaaaaagaaaaataatgagggctgtatggaatacattcattattcattcactca
ctcactcactcattcattcattcattcaacaagtcttattgcataccttctgtttgctca
gcttggtgcttggggctgctgaggggcaggagggagagggtgacatgggtcagctg
actcccagagtccactccctgtaggtcgggcagcaggccgtagaagtctggcaggg
cctggccctgctgtcggaagctgtcctgcggggccaggccctgttggtcaactcttccc
agccgtgggagcccctgcagctgcatgtggataaagccgtcagtggccttcgcagc
ctcaccactctgcttcgggctctgggagcccaggtgagtaggagcggacacttctgct
tgcccttctgtaagaaggggagaagggtcttgctaaggagtacaggaactgtccgta
ttccttccctttctgtggcactgcagcgacctcctgttttctccttggcagaaggaagccat
ctcccctccagatgcggcctcagctgctccactccgaacaatcactgctgacactttcc
gcaaactcttccgagtctactccaatttcctccggggaaagctgaagctgtacacagg
ggaggcctgcaggacaggggacagatg<u>a</u>ccaggtgtgtcc***aagctt*** ----------

The first translated atg codon, as well as the tga "stop" codon are underlined. The sequences of restriction sites utilized in the cloning are shown in bold italics.----------

2) GENETIC CONSTRUCTIONS, according to claim 1, characterized because the expression genetic systems consist in two vectors that have as control elements of the EPO expression only the early promoter of the SV40 virus and its terminator. and that allow to produce surprisingly high amounts of EPO, higher than 50/mg/ml/day, in CHO cells stably cotransfected with these vectors and selected according to their resistance to increasing quantities of MTX.----------

3. GENETIC CONSTRUCTIONS, according to claim 1, characterized because the EPO thus obtained consists in 165 amino acids according to the following sequence:----------

NH_2--- Ala Pro Pro Arg Leu Ile Cys Asp

Ser	Arg	Val	Leu	Glu	Arg	Tyr	Leu
Leu	Glu	Ala	Lys	Glu	Ala	Glu	***Asn***
Ile	Thr	Thr	Gly	Cys	Ala	Glu	Hys
Cys	Ser	Leu	Asn	Glu	***Asn***	Ile	Thr
Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val
Gly	Gln	Gln	Ala	Val	Glu	Val	Trp
Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu
Ala	Val	Leu	Arg	Gly	Gln	Ala	Leu
Leu	Val	***Asn***	Ser	Ser	Gln	Pro	Trp
Glu	Pro	Leu	Gln	Leu	Hys	Val	Asp
Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser
Leu	Thr	Thr	Leu	Leu	Arg	Ala	Leu
Gly	Ala	Gln	Lys	Glu	Ala	Ile	Ser
Pro	Pro	Asp	Ala	Ala	***Ser***	Ala	Ala
Pro	Leu	Arg	Thr	Ile	Thr	Ala	Asp
Thr	Phe	Arg	Lys	Leu	Phe	Arg	Val
Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys
Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Ala
Cys	Arg	Thr	Gly	Asp----COOH		,----------	

------ ***X***: glycosilation sites.----------

There follows an illegible signature followed by a seal that reads: HUMBERTO M. DE PASQUALE. ATTORNEY.----------

IX. Abstract.----------

The gene encoding for human erythropoietin (EPO) was obtained from human genomic DNA. This gene does not include homologous genetic elements located at 5' from the first translated ATG codon. The gene was cloned in an expression plasmid for eukaryotic cells. Hamster Ovary Cells (CHO) were cotransfected with this vector and other plasmid

expressing for dehydrofolatereductase (DHFR). Both plasmids have as unique expression control elements the early promoter of the SV40 virus and its polyadenylation signal. This system allows a double selection of the recombinant cells, as it confers resistance to geniticine and to methotrexate (MTX). Once selection was made, genetic amplification with MTX was performed. Productive capacity of the selected recombinant cells, was tested by means of an immunoassay specific for EPO----------

TECHNICAL DATASHEET----------

(10) PUBLICATION No.: AR----------

There appears a seal that reads I.N.P.I. 6 Nov 1998 12 37.---

Reception Desk.----------

(21) APPLICATION No.:----------

On the left, there appears the Argentinean Coat of Arms -------

(19) ----------

(51)INT. CL:----------

I.N.P.I.----------

Argentine Republic----------

P98 0105609.----------

(12) X INVENTION PATENT----------UTILITY MODEL----------

(2) SUBMISSION DATE: ----------

(30) PRIORITY DATA:----------

(41) APPLICATION PUBLICATION DATE: ----------

BULLETIN No.:----------

(61) ADDITIONAL TO: ----------

(62) DIVISIONAL FROM:----------

(71) APPLICANT: BIO SIDUS S.A.----------

(72) Inventor(s):----------

(74) Agent. 611 ----------

(83) Microorganism deposit: ----------

(54) TITLE OF THE INVENTION: "GENETIC CONSTRUCTIONS, CLONING METHOD FOR THE ENCODING GENE FOR RECOMBINANT HUMAN ERYTHROPOIETIN, SELECTION OF PRODUCING CELL LINE AND CELL CULTURE FOR THE MASS PRODUCTION OF RECOMBINANT HUMAN ERYTROPOIETIN."----------

(57) ABSTRACT:----------

The encoding gene for recombinant erythropoietin (EPO) was obtained from human genomic DNA. This gene does not include homologous genetic elements located at the 5´ of the first ATG translated codon. It was cloned in an expression vector for eukaryotic cells. Chinese Hamster Ovary (CHO) cell were co-transfected with this vector and other expression plasmid for dehydrofolactoreductase (DHFR). Both plasmids have the early promoter of SV40 virus and its polyadenylation signal. This system allows a double selection of recombinant cells, since they confer a geniticine and methotrexate (MTX) resistance. Once the selection is performed, gene amplification was done with MTX. Testing of the producing capacity of the chosen recombinant cells was performed with a specific immunoassay for EPO. ----------

Argentine Republic. There appears an Argentinean Coat of Arms. I.N.P.I.----------

APPLICATION OF----------

INVENTION PATENT: X----------

UTILITY MODEL CERTIFICATE----------

There appears a seal that reads INPI. 6 Nov. 1998. Reception Desk----------

FILING DATE.----------

Proceedings No.----------

I. Applicant ----------

(1) Name and Surname / Company´s Name:----------

BIO SIDUS S.A.----------

2). Identity Document:----------

Marital Status: ----------

Marriage: ----------

Spouse's Name: ----------

3) Retirement Account: ----------

No of CUIL (Unique Labor Identification Code) or CUIT (Unique Tax Identification Code): 30-59811709-4. ------ V.A.T: Liable for V.A.T Registration.----------

4) Registered in the National Industrial Registry (Decree-Law 19.971/72) No.-Does not apply.----------

5) Real Address: Constitución 4234. Buenos Aires. Argentina.-Legal Address: Alsina 971 – 1º Piso, of. "10, Buenos Aires ----

II. OBJECT: ----------

6) Title of invention: "Genetic constructions, cloning method for the encoding gene for recombinant human erythropoietin, selection of producing cell lines and cell culture for the mass production of recombinant human erythropoietin.----------

7) Type of patent: ----------

a) Final:for a 20-year period----------

b) Additional to Patent No.: ----------

8) Act 17,011. Priority Date: ----------

Country: ----------

No. ----------

III. Attached Documents ----------

(9) It is attached: ----------

a) Fee receipt for the requested service ----------

b) Form in duplicate ----------

c) Cover in duplicate ----------

d) Descriptive Memory in duplicate ----------

e) Signed claims in duplicate ----------

f) 2 copies of the first claim ----------

g) Diagrams in triplicate ----------

h) Number of boards ----------

i) Reduced-scale copies----------

j) Certified copy (Act 17,011)----------

k) Assignment Document ----------

l) Draft drawings----------

IV Legal Entities ----------

10) The corporation, represented by HUMBERTO MARIO DE PASQUALE----------

who state under oath that he is the attorney with his powers in force and that the corporation is registered in the Public Commerce Registry on Date: 10/07/1983 No. 7258. Page: -- Book: 98. Volume: A.----------

V. Power of Attorney----------

11) Power of Attorney registered in ----------Registered in the INPI (National Institute of Industrial Property) under No.: Other Registry: ---------No.----------

12) In this case,: CARLOS MIGUEL COLL ARECO and/or HUGO EDUARDO MARTINEZ LAHITOU are authorized to proceed in this matter until its finalization with the power of signing documents, waving, if necessary, and requesting certificates.----------

13) Power of Attorney is attached----------

14) Retirement Account / Company: CONSOLIDAR No. CUIL or CUIT: CUIT 20-04991729-6 CUIT: 20-16821007-9----------

15) Agent No. 611/900.----------

VI Statement ----------

16) In view of the Provision with no number dated 7 June 1901 (on Patentability in foreign countries) it is declared that the invention has not been patented abroad----------

VII. Remarks: According to Article 19 Act 24,481 and its Regulatory Decree, complementary information will be supplied within the legal term.----------

There appears a signature. Carlos Miguel Coll Areco (Signature of the authorized person). ----------

There appears a signature. Humberto Mario De Pasquale. Attorney. Signature of applicant----------

There appears the Argentinian Coat of Arms----------

MINISTRY OF ECONOMY AND PUBLIC SERVICES----------

NATIONAL INSTITUTE OF INDUSTRIAL PROPERTY----------

INVENTION PATENT----------

February 23 1999----------

Reception Desk----------

National Institute of Industrial Property----------

Application of: Invention Patent----------File:----------

Under the proceedings P98 01 05609 an INVENTION PATENT application has been entered----------

Buenos Aires, (blank)199--.... ----------

INPI 6 Nov. 1998. Reception Desk.----------

Date: 11/06/1999----------Time: 12:37----------

Resp: Coll Areco, Carlos Miguel----------P19990100679--

File Bar Code----------Query Priority Order--

01996010560906111237----------P19980105609-

I, VIVIANA BEATRIZ CUMBO, A SWORN PUBLIC TRANSLATOR, DO HEREBY CERTIFY THE FOREGOING TO BE A TRUE TRANSLATION INTO ENGLISH OF THE PHOTOCOPY OF THE ORIGINAL DOCUMENT IN SPANISH LANGUAGE, ATTACHED HERETO, WHICH I HAVE HAD BEFORE ME. DONE AND SIGNED IN BUENOS AIRES, ON THIS FOURTEENTH DAY OF NOVEMBER, TWO THOUSAND AND THREE.----------

YO, VIVIANA BEATRIZ CUMBO, TRADUCTORA PÚBLICA MATRICULADA, CERTIFICO POR LA PRESENTE, QUE ÉSTA ES TRADUCCIÓN FIEL AL IDIOMA INGLÉS DE LA FOTOCOPIA DEL DOCUMENTO ORIGINAL REDACTADO EN IDIOMA CASTELLANO, ADJUNTA A LA PRESENTE, QUE HE TENIDO A LA VISTA, Y A LA CUAL ME REMITO, EN BUENOS AIRES, A LOS CATORCE DÍAS DEL MES DE NOVIEMBRE DE DOS MIL TRES.----------